RePlex™ Method Development Guide

protein simple

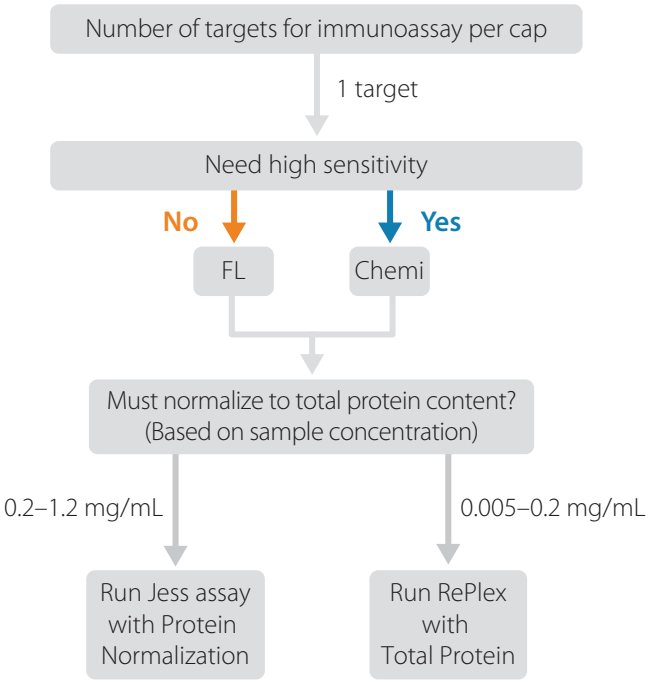
Introduction

Jess™ enables detection of proteins using fluorescence and chemiluminescence, but also provides the added benefit of two different methods of protein normalization. A successfully optimized method gives you reliable and quantitative data. This guide focuses on method development for RePlex™ assays, which allow running either two sequential immunoassays or an immunoassay followed by the Total Protein Assay in a single run. This is done in two different probing cycles (Probe 1 and Probe 2).

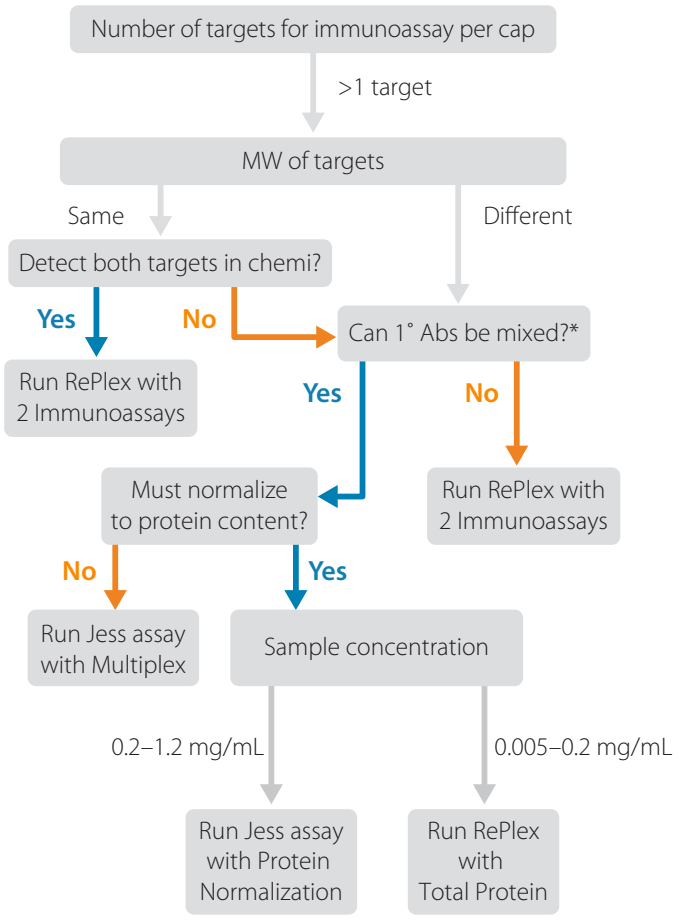
This guide assumes that you have already optimized detection for your target protein on Jess. For more information on how to do this, refer to the "Simple Western Size Assay Development Flowchart".

Jess provides multiple options for detecting multiple targets and different ways to perform total protein normalization. While this guide will focus on developing a RePlex assay, a flowchart on when to use RePlex versus other assay types for Jess is provided below:

Overview of Assay Selection – only 1 target protein



Overview of Assay Selection – more than 1 target protein



RePlex: Recommend probing target with lower chemiluminescence signal or using fluorescence detection in Probe 1 if using chemiluminescence detection in Probe 2.

Multiplex: Assay where multiple primary antibodies are mixed together and, if applicable, multiple secondary antibodies are mixed together.

*Check antibody cross-reactivity, dynamic range, and background levels for each antibody to be mixed.

Two Immunoassays

For RePlex™ assays where antibodies for two target proteins or two antibodies for the same target are used across the two probing cycles, it is recommended to probe either the lower abundant protein or the more labile post-translational modification in Probe 1.

When performing RePlex assays, the optimized conditions determined for each antibody individually should be used. For quantitative results, both assays must perform within the same linear range at the desired lysate concentration used in the assay. For additional information on the dynamic ranges for chemiluminescence and fluorescence on Jess™, please see the "Multiplexed Western Blotting Redefined: Superplexing on Jess" application note (PL7-0047). Additionally, when using chemiluminescence detection, the antibody used should have a stable signal across all exposures. Viewing the Graphs in Compass for Simple Western using the All Exposures option is an easy and quick way to identify signal decay (**Figure 1A**). The antibody used in Probe 1 should also not have an extremely high baseline or peak signal that shows significant signal decay with increasing exposure times (**Figure 1B**). An example of an ideal target without signal decay is shown in **Figure 1C**. Note that the peak signal overlays well across the exposure series.

When setting up your first RePlex assay using two optimized antibodies, we recommend including controls in the assay, which are shown in **Figure 2**, as part of the plate layout. These controls allow you to determine the optimal probing order and antibody removal efficiency. See **Figure 4** for additional controls to perform when detecting two targets at the same molecular weight (MW) using chemiluminescence in both probing steps.

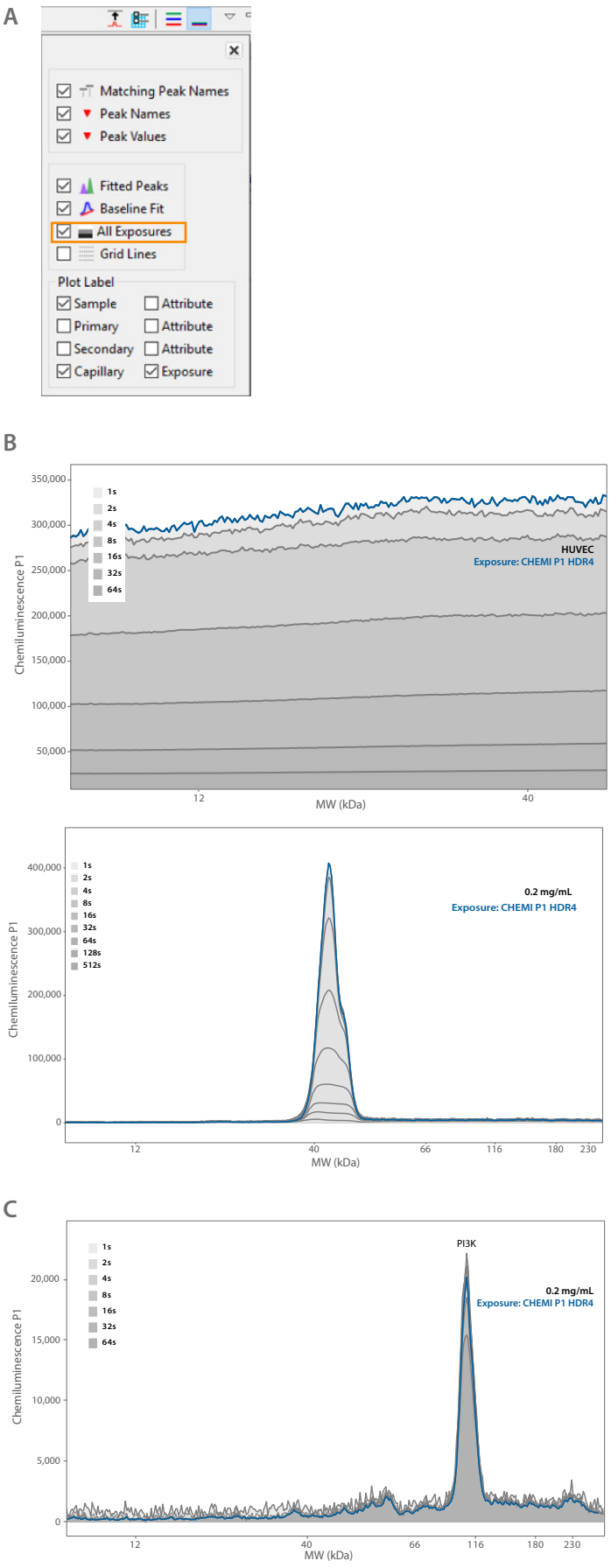


FIGURE 1. Using the All Exposures view within the Graph Options in Compass for Simple Western (A) is useful for identifying signal decay. An antibody with high baseline (top) or high peak signal (bottom) may show signal decay (B) when comparing the baseline value or peak signal across exposures. An antibody with stable signal over an exposure series will have multiple exposures overlapped (C).

CAPILLARY/PLATE WELL

PLATE ROW	1	2	3	4	5	6	7
A	Biot. Ladder	Sample 1					
B	Antibody Diluent						
C	Blocking	Primary A	Primary B	Antibody Diluent		Primary A	Primary B
D	Streptavidin	Secondary A	Secondary B	Antibody Diluent		Secondary A	Secondary B
E	Blocking	Primary B	Primary A		Primary B	Antibody Diluent	
F	Blocking	Secondary B	Secondary A		Secondary B	Secondary A	Secondary B
Objective		Determine antibody probing order		Control for Probe 1 effect on Probe 2; Determine primary antibody stability		Determine antibody removal efficiency	

FIGURE 2. Overview of initial RePlex assay layout when optimizing for detection of two target proteins. This layout can be applied three times across an assay plate, allowing for optimization of three target/antibody pairs.

DETERMINING PROBING ORDER AND CHECKING PRIMARY ANTIBODY STABILITY

After running the assay shown in **Figure 2**, antibody probing order is determined by reviewing the data as follows:

- Compare signal in **capillaries 2** and **4** for **Primary antibody A**. If signal for this antibody is lower in capillary 4, then the antibody should be used in Probe 1. If signal for this antibody is similar in both capillaries, then it can be used in either Probe 1 or Probe 2.
- Compare signal in **capillaries 3** and **5** for **Primary antibody B**. If signal for this antibody is lower in capillary 5, then the antibody should be used in Probe 1. If signal for this antibody is similar in both capillaries, then it can be used in either Probe 1 or Probe 2.

***NOTE:** Occasionally, higher signal is observed in Probe 2 for a given antibody. For RePlex™ assays, it is always recommended to use a particular antibody in a single probing step (Probe 1 or 2) when comparing different samples. Do not quantitate or compare signal for the same antibody across probes!*

IDENTIFYING PROBE 1 EFFECTS ON PROBE 2

After running the assay shown in **Figure 2**, any effects on Probe 2 resulting from the immunoassay performed in Probe 1 are identified by reviewing the data as follows:

- Compare signal in **capillaries 3** and **4** for **Primary antibody A**. If signal for this antibody is lower in capillary 3, then the antibody should be used in Probe 1. If signal for this antibody is similar in both capillaries, then it can be used in either Probe 1 or Probe 2.
- Compare signal in **capillaries 2** and **5** for **Primary antibody B**. If signal for this antibody is lower in capillary 2, then the antibody should be used in Probe 1. If signal for this antibody is similar in both capillaries, then it can be used in either Probe 1 or Probe 2.

CALCULATING ANTIBODY REMOVAL EFFICIENCY

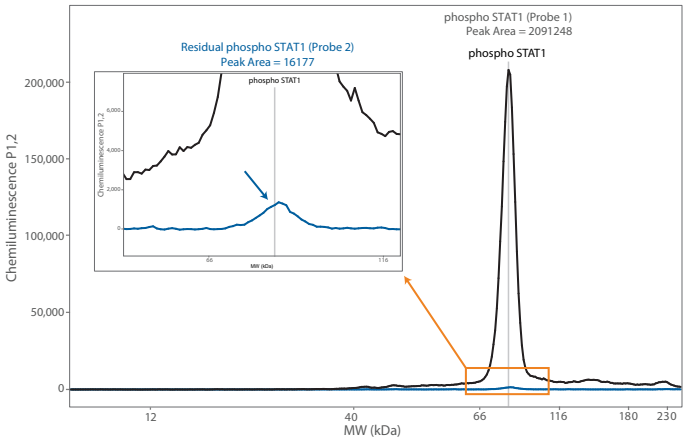
It is important to verify the antibody removal efficiency achieved between Probe 1 and Probe 2, as this can be antibody dependent. If residual antibody from Probe 1 is carried into the Probe 2 step, and the secondary antibody for Probe 2 is the same species or not cross-adsorbed, carry-over signal can be expected and will interfere with accurate quantitation of Probe 2. Antibody removal efficiency is determined by re-probing with the same secondary antibody in Probe 2 after the primary and secondary antibodies from Probe 1 are removed (capillaries 6 and 7 in **Figure 2**). This detects any residual primary antibody from Probe 1. It can be calculated as follows:

$$\frac{(\textit{Peak Area Probe 1} - \textit{Peak Area Probe 2})}{(\textit{Peak Area Probe 1})} \times 100 = \textit{Antibody Removal Efficiency (\%)}$$

An example of calculating antibody removal efficiency for phospho STAT1 antibody is shown in **Figure 3**. An antibody removal efficiency of at least 95% should be expected. If this is not achieved for a given antibody, then the antibody should only be used in Probe 2 of a RePlex™ assay.

USING CHEMILUMINESCENCE FOR TWO TARGETS AT THE SAME MW

RePlex assays allow chemiluminescent detection of two targets at the same MW by probing each target in separate probes. One of the most common use cases for this assay is detection of phosphorylated and total protein for a given target when combining the antibodies in the same incubation for a two-color Jess™ detection is not possible, such as when both primary antibodies are the same species. For these types of assays, we recommend the following controls and plate setup, summarized in **Figure 4**. For this experiment, use a sample that is expected to have phosphorylated protein. Additional lysate concentrations are included to more quickly identify optimal assay conditions based on the results from this initial experiment. These dilutions should be serial dilutions of 2–3 fold changes in lysate concentration. The same controls as in **Figure 2** are included in this plate map and can be used to determine probing order, possible effects



$$\frac{(2091248-16177)}{2091248} \times 100 = 99.2\%\ \textit{Antibody Removal Efficiency}$$

FIGURE 3. Calculating antibody removal efficiency for phospho STAT1. The Graph view shows Probe 1 and Probe 2 overlaid, with a zoomed-in view of Probe 2 (inset). Antibody removal efficiency is calculated using the peak areas from Probe 1 and Probe 2.

CAPILLARY/PLATE WELL

PLATE ROW	1	2	3	4	5	6	7	8	9	10	11	12	13
A	Biot. Ladder	Low lysate concentration		Mid-low lysate concentration		High-mid lysate concentration		High lysate concentration					
B	Antibody Diluent												
C	Blocking	Phospho Ab (X)	Total Ab (Y)	Phospho Ab (X)	Total Ab (Y)	Phospho Ab (X)	Total Ab (Y)	Phospho Ab (X)	Total Ab (Y)	Antibody Diluent		Phospho Ab (X)	Total Ab (Y)
D	Streptavidin	Secondary X	Secondary Y	Secondary X	Secondary Y	Secondary X	Secondary Y	Secondary X	Secondary Y	Antibody Diluent		Secondary X	Secondary Y
E	Blocking	Total Ab (Y)	Phospho Ab (X)	Total Ab (Y)	Phospho Ab (X)	Total Ab (Y)	Phospho Ab (X)	Total Ab (Y)	Phospho Ab (X)		Total Ab (Y)	Antibody Diluent	
F	Blocking	Secondary Y	Secondary X	Secondary Y	Secondary X	Secondary Y	Secondary X	Secondary Y	Secondary X		Secondary Y	Secondary X	Secondary Y
Objective		Determine antibody probing order **Low lysate**		Determine antibody probing order, **Mid-low lysate**		Determine antibody probing order, **High-mid lysate**		Determine antibody probing order, **High lysate**		Control for Probe 1 effect on Probe 2; Determine primary antibody stability		Determine antibody removal efficiency	

FIGURE 4. Plate setup for optimizing a RePlex assay using antibodies to detect phosphorylated and total protein. This layout can be applied two times across an assay plate, allowing for optimization of two target/antibody pairs.

of Probe 1 on Probe 2, and antibody removal efficiency as described previously in this guide.

DETERMINING OPTIMAL LYSATE CONCENTRATION

The plate layout in **Figure 4** helps to identify the lysate concentration to use in your optimized RePlex™ assay. Use the All Exposures view to check for signal decay in Probe 1 for the highest lysate concentration samples (capillaries 8 and 12 for Phospho antibody and capillaries 9 and 13 for Total antibody).

- If only one antibody shows signal decay at short exposure times (less than 16 seconds), then that antibody should be used in Probe 2.
  - ***Quantitation tip:*** It is recommended to use lysate concentrations that do not show signal decay.
- If both antibodies show signal decay at short exposure times (less than 16 seconds), then a lower lysate concentration should be used. Check capillaries with lower lysate concentrations (capillaries 2 – 7) and choose the concentration where both targets are detected. **Figure 5** shows an example of how lower lysate concentrations reduce signal decay.

When a lower lysate concentration cannot be used to detect both targets:

- Use NIR detection in Probe 1 for the target with the highest signal and the secondary target is detected with chemiluminescence in Probe 2.
  - ***Quantitation tip:*** It is recommended to do a follow-up run to confirm that the NIR signal is in the linear range for the higher signal target.
- Use a single chemiluminescence exposure setting in the RePlex assay for Probe 1. Choose the longest chemiluminescence exposure where the signal is stable as seen in the All Exposures view. In the RePlex assay, uncheck the **RePlex Dynamic Range** in the Detection Profile in Compass for Simple Western and manually input the desired exposure time (**Figure 6**).
- Identify an alternate primary antibody to replace the antibody that shows signal decay.
  - ***Quantitation tip:*** It is recommended to do a follow-up run to confirm that the new antibody signal is in the linear range for the desired lysate concentration.

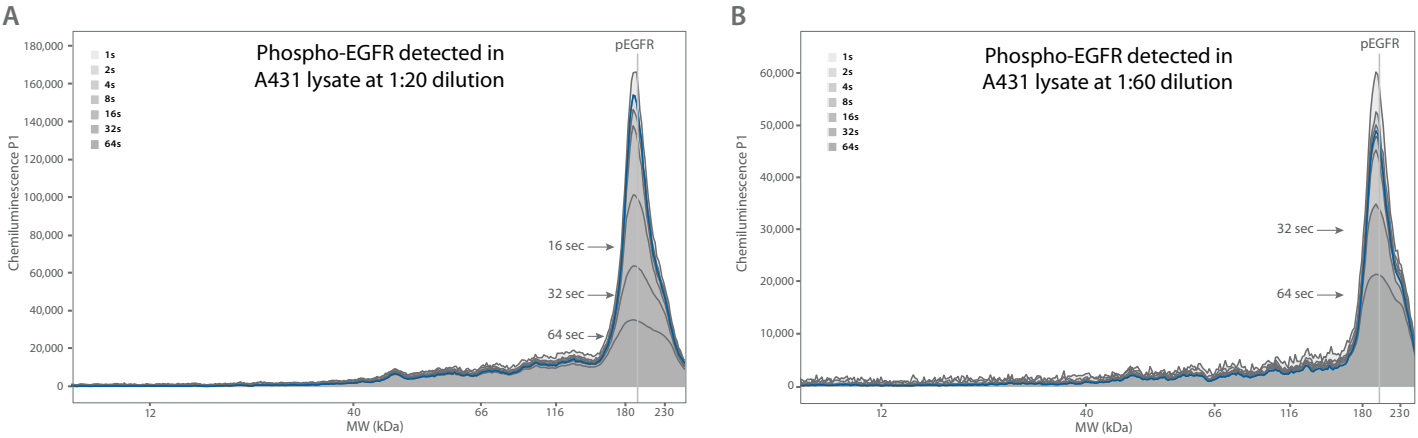


FIGURE 5. Reducing lysate concentration results in reduction of signal decay for chemiluminescence. Detection of phosphorylated EGFR in A431 lysates at a 1:20 dilution results in signal decay as observed in the All Exposures view in Compass for Simple Western (A). Reducing the sample concentration by using a 1:60 dilution of the lysate results in a more stable signal as indicated by more overlapping exposures (B).

IDENTIFYING CHEMILUMINESCENCE-INDUCED DAMAGE FOR PROBE 2 TARGETS AT THE SAME MW AS PROBE 1 TARGETS

In chemiluminescent immunoassays, the horseradish peroxidase (HRP) on the secondary antibody produces highly concentrated and localized free radicals during the reaction with luminol, in addition to the light that is detected as the signal in the assay[1]. In conditions where an abundance of free radicals are produced in Probe 1, these radicals can damage nearby antigens that are recognized by an antibody used in Probe 2. Thus, in RePlex™ chemiluminescence assays, the signal for the target in Probe 2 can be affected if it is at the same MW as the target detected in Probe 1.

Using the plate setup in **Figure 4** and the total/phospho antibody model as an example, to identify if damage has occurred, compare the total antibody signal in Probe 2 for capillaries 8 and 11. If the signal in capillary 8 is significantly lower (> 20%), it is likely that the phospho antibody used in Probe 1 had a high enough signal to result in antigen damage. **Figure 7** shows an example of this effect, where the signal for c-Jun in 3T3 lysates is much lower for capillary 8 than in capillary 11.

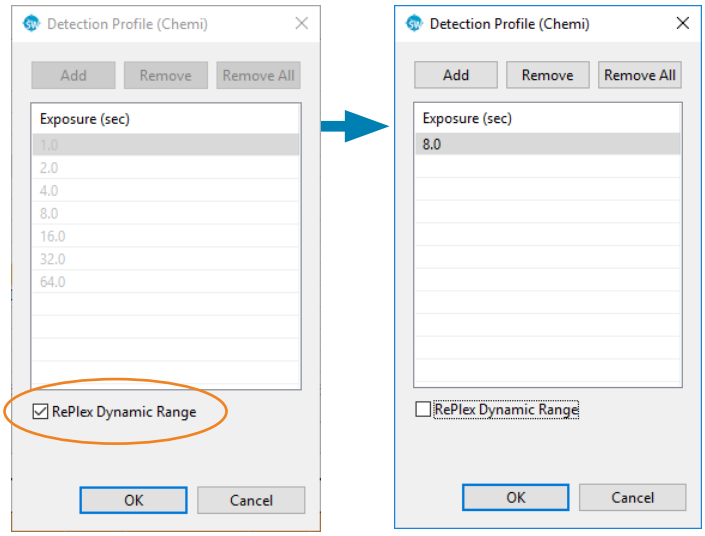


FIGURE 6. Changing to single exposure times in RePlex chemiluminescence assays in Compass for Simple Western.

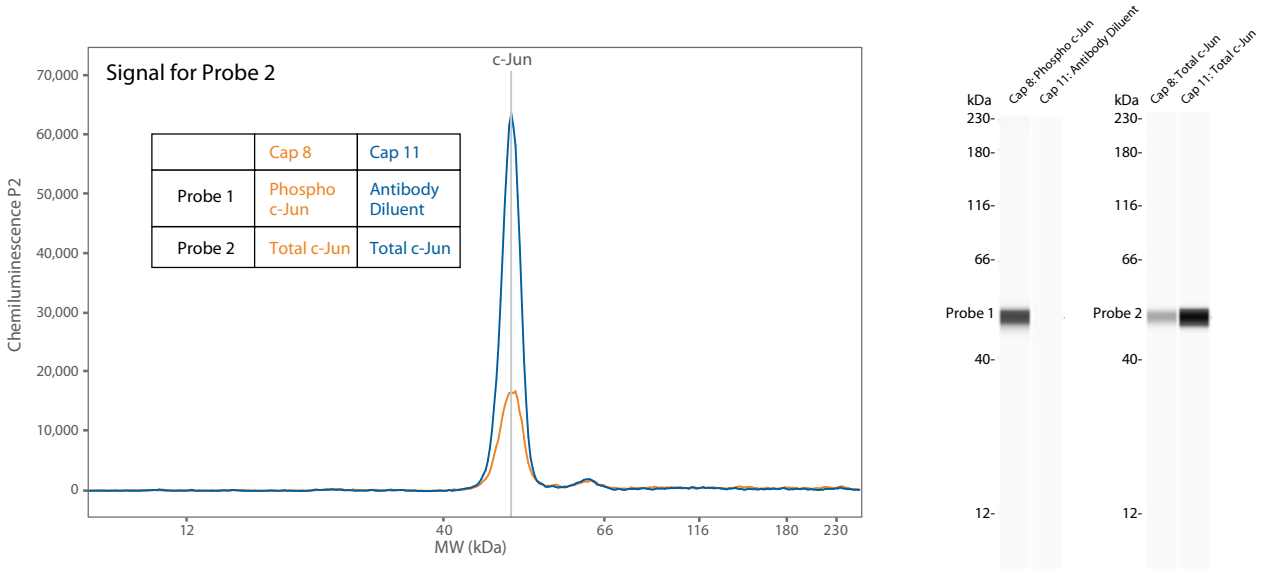


FIGURE 7. Reduction in c-Jun signal in Probe 2 due to chemiluminescence detection of phosphorylated c-Jun in Probe 1 in 3T3 lysates. When running the plate layout as shown in **Figure 4**, the signal for total c-Jun (Probe 2) in capillary 8 is much lower than in capillary 11 (left), suggesting that detecting phosphorylated c-Jun in Probe 1 should be avoided when using high 3T3 lysate concentrations in RePlex. Lane view (right) shows the signals for Probe 1 and Probe 2.

- If chemiluminescent damage is observed for a target detected in Probe 2 AND you are unable to use a different probing order, then a lower lysate concentration should be used. Check capillaries with lower lysate concentrations (capillaries 2 – 7) and choose the concentration where both targets are detected.

When a lower lysate concentration cannot be used to detect both targets:

- Use NIR detection in Probe 1 for the target with the highest signal. The second target is detected with chemiluminescence in Probe 2.
  - ***Quantitation tip:*** It is recommended to do a follow-up run to confirm that the NIR signal is in the linear range for the higher signal target.
- Use a single chemiluminescence exposure setting in the RePlex™ assay for Probe 1. Choose the longest exposure where the chemiluminescence signal is stable as seen in the All Exposures view. In the RePlex assay, uncheck the **RePlex Dynamic Range** in the Detection Profile in Compass for Simple Western and manually input the desired exposure time (**Figure 6**).
- Identify an alternate primary antibody to replace the antibody that shows signal decay.
  - ***Quantitation tip:*** It is recommended to do a follow-up run to confirm that the new antibody signal is in the linear range for the desired lysate concentration.

Immunoassay with Total Protein

With RePlex, the chemiluminescent Total Protein Assay, which is used with the Total Protein Detection Module (P/N DM-TP01), can be run in combination with an immunoassay to allow for normalization based on total protein signal. The Total Protein Assay is always performed in Probe 2 in a RePlex assay and works best with lysate protein concentrations that range between 0.2 mg/mL – 0.005 mg/mL. This concentration range will ensure better linearity for the Total Protein Assay. **Figure 8** shows the linear range for total protein signal using HeLa lysates is

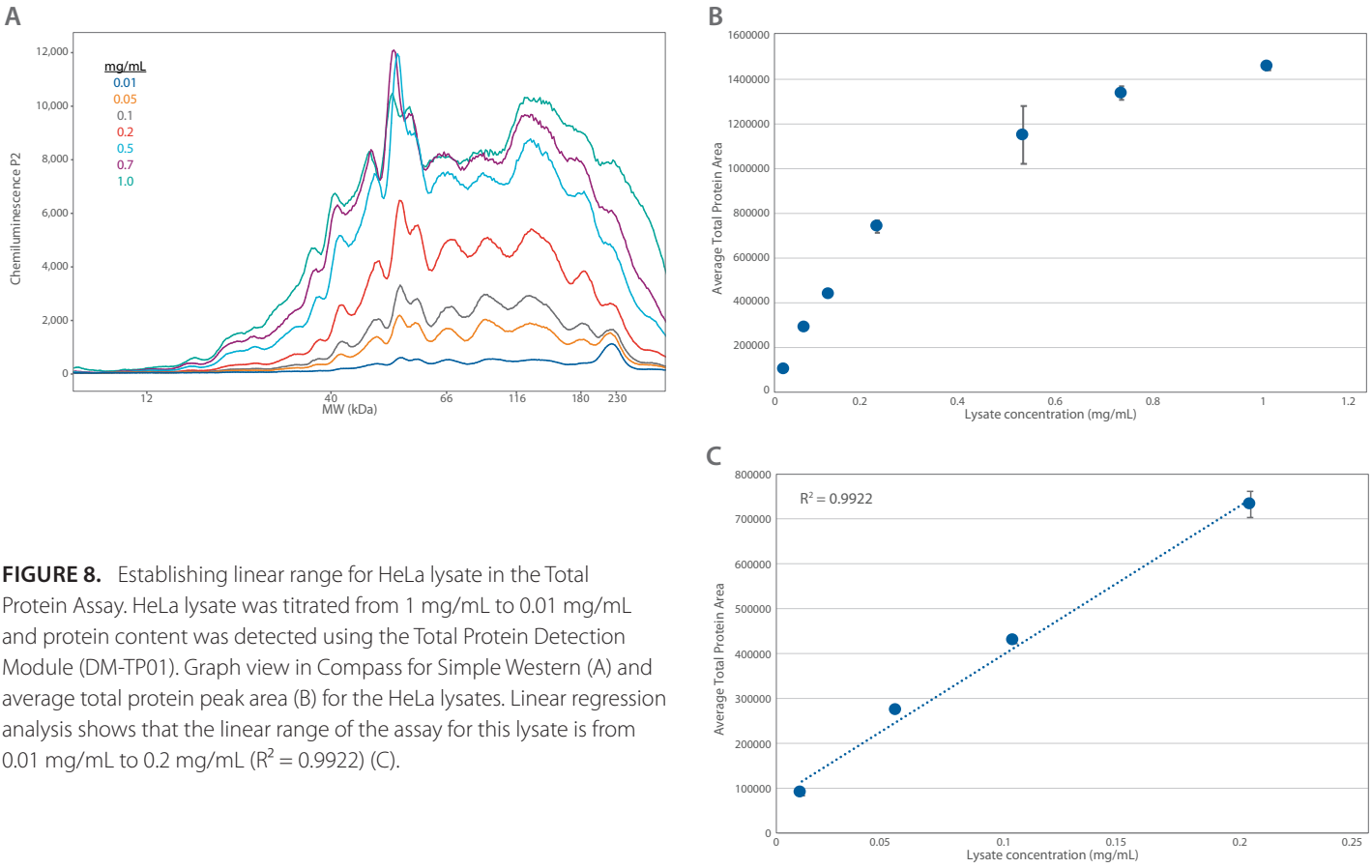


FIGURE 8. Establishing linear range for HeLa lysate in the Total Protein Assay. HeLa lysate was titrated from 1 mg/mL to 0.01 mg/mL and protein content was detected using the Total Protein Detection Module (DM-TP01). Graph view in Compass for Simple Western (A) and average total protein peak area (B) for the HeLa lysates. Linear regression analysis shows that the linear range of the assay for this lysate is from 0.01 mg/mL to 0.2 mg/mL ($R^2 = 0.9922$) (C).

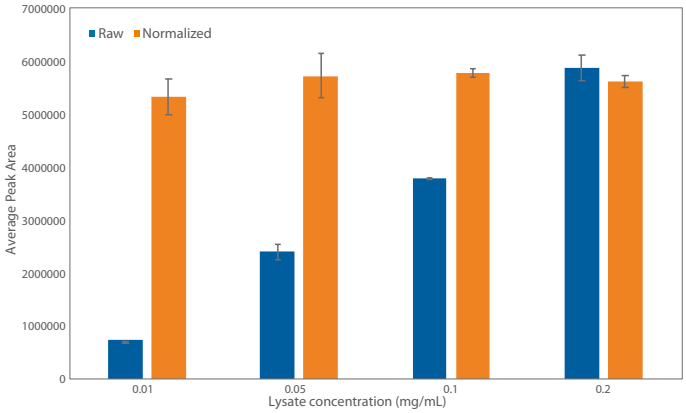


FIGURE 9. Protein normalization using RePlex shows comparable 14-3-3 signal across increasing concentrations of HeLa lysate. HeLa lysates (0.01 – 0.2 mg/mL) were separated and probed with a 14-3-3 antibody in Probe 1 followed by the Total Protein Assay in Probe 2. 14-3-3 expression is shown as raw peak area (blue bars) and normalized peak area (orange bars).

from 0.01 – 0.2 mg/mL as determined by linear regression analysis ($R^2 = 0.9922$). Once the linear range of the antibody and lysate are determined, a RePlex™ assay can be used to perform quantitative normalization. **Figure 9** shows an example of normalization of 14-3-3 signal in HeLa lysates using the highest lysate concentration as the reference for normalization. As expected, higher signals (average peak area) for 14-3-3 are detected with increasing lysate concentrations (blue bars). Normalizing the 14-3-3 signal to the total protein area measured in the RePlex assay resulted in similar peak area across all lysate concentrations (orange bars).

When running RePlex assays using the Total Protein Detection Module for protein normalization, it is also recommended that:

1. The target protein signal does not show signal decay (refer to **Figure 5**).
2. The immunoassay is in the linear range of the assay, where the amount of signal for the protein target displays a linear relationship with the amount of sample loaded in the assay plate.
3. The Biotin Labeling reagent is prepared immediately before loading into the plate.

References

1. The Necessity of and Strategies for Improving Confidence in the Accuracy of Western Blots. R Ghosh, J Gilda, and AV Gomes. *Expert Rev Proteomics*, 2014; 11(5): 549-560.

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